Abstract

Cytokinin is a plant hormone that plays key roles in regulating several aspects of growth and development, including nutrient uptake, cell division, shoot initiation, and leaf senescence. The framework of the cytokinin signal transduction pathway in plants involves a slightly modified version of a two-component phosphorelay system. Type-A *ARABIDOPSIS* RESPONSE REGULATORS (ARRs) are negative regulators of this pathway. A quadruple type-A *arr* loss-of-function mutant, *arr3,4,8,9*, is hypersensitive to cytokinin and displays a short root phenotype when subjected to low levels of cytokinin. In order to identify novel components of the cytokinin signaling pathway, mutagenized *arr3,4,8,9* progeny pools were screened for *suppressors of cytokinin hypersensitivity* (*sch*). To date, 376 putative *sch* mutants have been identified in the M_2. Of the 180 M_3 mutants tested, 89 exhibited longer roots and decreased sensitivity to low levels of cytokinin compared to the *arr3,4,8,9* parent. Future directions include continued confirmation of putative mutant lines and mapping of causative mutations for the suppressor phenotype using bulked segregant analysis. Identification of novel cytokinin signaling components may improve crop engineering efforts, as cytokinin signaling is involved in the development of several desirable plant traits, including enhanced drought resistance and increased crop yield.

Introduction

Cytokinin is a pleiotropic plant hormone that plays a key role in regulating several aspects of plant growth and development. It has been implicated in cell division, nutrient uptake, root and shoot elongation, responses to biotic and abiotic factors, and embryonic development (Kieber and Schaller 2014, Mok 1994). Recent studies have shown that manipulation of the cytokinin pathway can increase drought tolerance, enhance crop yields, and delay leaf senescence (Mok 1994, Reguera *et al.* 2013, Zaman *et al.* 2015, Zwack 2013). However, the effectors of the cytokinin signal in these developmental and physiological processes are still largely unknown, thereby limiting any current applications to genetic engineering (Hutchinson and Kieber 2002). Thus, the goal of this project is to identify new components that may play a role in the cytokinin signaling pathway.

The framework of the cytokinin signal transduction pathway in plants involves a slightly modified version of a prokaryotic two-component phosphorelay system (To *et al*. 2007). In plants, this pathway is initiated by binding of cytokinin to the CHASE domain of an *ARABIDOPSIS* HISTIDINE KINASE (AHK), which leads to autophosphorylation of a histidine residue contained within its transmitter domain. The phosphate group is then transferred to an aspartic acid in the receiver domain, and subsequently transferred to a histidine of an *ARABIDOPSIS* HISTIDINE PHOSPHOTRANSFER PROTEIN (AHP). Finally, upon phosphorylation, AHP moves into the nucleus and transfers the phosphate group to an aspartic acid residue contained on *ARABIDOPSIS* RESPONSE REGULATORS (ARRs), which are divided into two classes: type-A ARRs, which act as negative regulators of the cytokinin signaling pathway, and type-B ARRs, which activate transcription of cytokinin induced genes,

including the type-A ARRs (Figure 1) (To *et al.* 2004, To *et al.* 2007, Hutchinson and Kieber 2002).

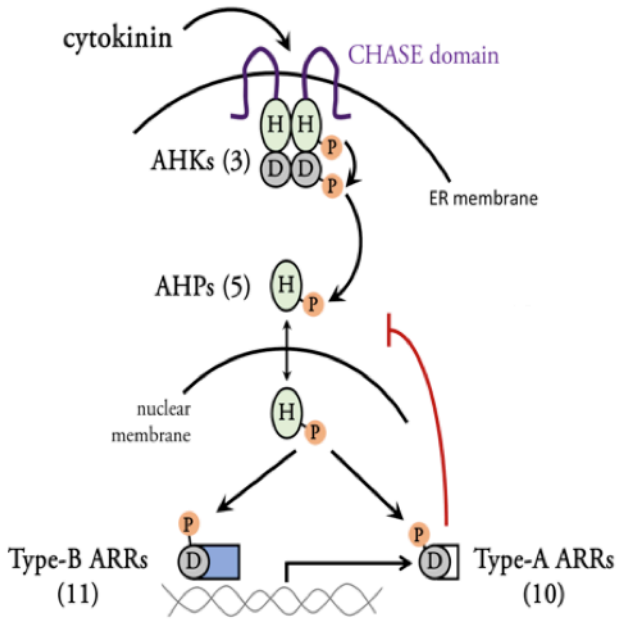

Figure 1. Cytokinin signal transduction pathway
Cytokinin is bound by the CHASE domain of an AHK, which results in autophosphorylation of a histidine residue in its transmitter domain. The phosphate is subsequently transferred to an aspartic acid in the AHK receiver domain and then to a histidine on an AHP, which shuttles the phosphate across the nuclear membrane. Once inside the nucleus, the phosphate is transferred to a type-A ARR or a type-B ARR, resulting in their activation (Figure adapted from Kieber and Schaller 2014).

Genetic screens for suppressors of a given phenotype are a powerful tool for dissecting signal transduction pathways (Li and Zhang 2016). Researchers have utilized this approach to successfully isolate several mutants in a diverse array of biological pathways in *Arabidopsis thaliana*. For instance, *BRI1-LIKE RECEPTOR KINASE* (*BRL1*), which encodes a leucine-rich repeat receptor-like protein kinase, was identified in a suppressor screen that utilized a *brassinosteroid insensitive 1 (bri1)* background, and it was revealed to play a partially redundant role with *BRI1* in the brassinosteroid signaling pathway (Zhou *et al.* 2004). Another example is the gain-of-function *ethylene response sensor 1* mutant *(ers1-4*), which restored hypocotyl growth of ethylene-treated *enhanced ethylene response mutant* (*eer5-1)* to wild-type levels and

led to the discovery of a novel regulatory role for *ERS1* in the ethylene signaling pathway (Deslauriers *et al.* 2007). Similarly, *MODIFIER OF SNC1,3 (MOS3)*, a gene that is crucial to basal resistance to pathogens in *Arabidopsis*, was discovered via a defense signaling suppressor screen that isolated suppressors of the *synaptobrevin homolog 1* (*snc1*) mutant phenotype (Zhang and Li 2005). Thus, suppressor screening is an established and effective method for identifying novel genes and functions involved in signal transduction pathways.

Cytokinin is a negative regulator of root elongation, so we employed a suppressor screen using the sensitized background of a quadruple type-A ARR mutant line, *arr3,4,8,9*. This mutant is hypersensitive to cytokinin and exhibits a short root phenotype in response to low concentrations of cytokinin, resulting from removal of negative feedback by the type-A response regulators in the cytokinin pathway (To *et al.* 2007). My project focused on screening for *suppressors of cytokinin hypersensitivity* (*sch*), or mutants that suppressed the short root phenotype of *arr3,4,8,9* seedlings in the presence of low levels of cytokinin. To date, I have identified 376 putative M_2 mutants. Future research will include retesting M_3 populations, identifying cytokinin insensitive mutants or mutants involved in overlapping pathways, mapping causative mutations, and determining the roles that these putative *sch* mutants play in the cytokinin signaling pathway.

Materials and Methods

All reagents utilized in this study were obtained from Sigma-Aldrich (St. Louis, MO) unless stated otherwise.

Ethyl Methanesulfonate (EMS) Mutagenesis

Seed (1g) from the parental line, *arr3,4,8,9*, were subjected to EMS treatment (Li and Zhang 2016). The seeds were suspended in a 15 mL solution containing 7.7 mM Na_2HPO_4, 92

mM NaH_2PO_4, and 5% (v/v) dimethyl sulfoxide, pH = 7.5. EMS was added to a final concentration of 20 mM. The seed was transferred to a fume hood and shaken for 16 hours to ensure equal exposure to EMS treatment. It was then washed three times with 100 mM Na_2SO_3, and three times with sterile deionized water. The M_1 seed were sprinkled onto 200 flats of soil and grown in a greenhouse. Approximately 10,000 M_1 plants germinated in total. The M_2 seed from each flat were pooled and harvested, yielding 200 M_2 pools for screening.

Primary Screen

M_2 pools were sterilized by taking approximately 0.1 mL of seed from each pool and surface-sterilizing with a 70% (v/v) ethanol (Decon Laboratories) wash. The ethanol was decanted and the seed were subsequently washed with a seed sterilization solution (20% (v/v) bleach (Clorox), 0.1% (v/v) Tween20 (Fisher Scientific)) for 5 minutes. The solution was decanted and washed with sterile deionized water 3-4 times. M_2 seed was densely plated in three evenly spaced rows on 0.5x Murashige Skoog (MS) phytagel plates containing 10 nM benzyl adenine (BA) (Figure 4). These plates consisted of 4g/L of (MS) salts containing 2.6 mM 2-(N-morpholino)ethanesulfonic acid buffer (Research Products International), 10% (w/v) sucrose, and 6g/L phytagel; the solution was pH adjusted to 5.8 and autoclaved before the addition of 10 nm BA. Seed was stratified for two days, and then transferred to a Percival growth chamber with fluorescent light (24 hour light; 22°C). Plants grew for eight days on the 10 nM BA plates before they were screened. Mutants that exhibited a long root phenotype were scored on a one to five-star system based on the degree of length, with the lines with the longest roots being scored as five. From each pool, these putative mutants were then transferred to soil and moved to an 18-hour photoperiod growth room under fluorescent lighting until they flowered. The M_2 plants were allowed to self-pollinate and M_3 seeds were harvested.

Retesting Putative Mutants (Secondary Screen)

The M_3 putative mutant seed was grown on 10 nM BA plates along with the original parent line, *arr3,4,8,9.* The seed was sterilized and stratified as was done in the primary screen. Twelve M_3 putative mutant seed were sowed on one half of a 10 nM BA plate and twelve *arr3,4,8,9* seed were sowed on the other half as a control. They were placed in the same Percival growth chamber with the same settings as was used for the primary screens. Root length was noted at the three-day mark to normalize for seed germination times. The plants were then allowed an additional five-day growth period before being analyzed at the eight-day mark. Each of the plates was scanned with a desktop scanner (Epson) and root lengths were determined using ImageJ software. Utilizing code written by the lab in R, average root lengths for the negative control and the putative mutants were determined at the eight-day mark. Root elongation assays were conducted to test for significance (2 sample t-test, $\alpha = 0.05$).

Cytokinin Insensitivity Test

In order to rule out previously identified cytokinin signaling genes that may confer cytokinin insensitivity, mutants with the most significant p-values ($p < 10^{-3}$) were retested on 1 μm BA plates using the secondary screen protocol described above. For this test, a cytokinin insensitive mutant, *ahk4*, was utilized as the control. Mutants that exhibited cytokinin insensitivity at 1 μM BA were excluded from further analyses.

Triple Response Assays

In order to rule out ethylene signaling pathway mutants, plants that were cytokinin sensitive at 1 μM BA were rescreened on 10 μM 1-Aminocyclopropane-1-carboxylic acid (ACC). About 50 seeds from each mutant were sowed onto one-fourth of a 10 μM ACC plate, and *ethylene insensitive 2 (ein2)*, wild-type, *arr3,4,8,9*, and *ahk4* served as controls. Plates were

wrapped in tin foil, stratified for two days, unwrapped, and then light-treated in a Percival growth chamber for three hours under the same settings as was used in the primary screen. The plates were then rewrapped with tin foil, placed in a black bag, and allowed to grow at 22°C in a dark incubator for three days. Mutants that did not exhibit ethylene sensitivity, also known as the triple response, were excluded from mapping.

Cetrimonium Bromide (CTAB) DNA Extraction for Library Preparation

Plant tissue (1 cm^2) was harvested in 1.5 mL microcentrifuge tubes and 2-4 metal beads were placed in each tube. The tubes were flash frozen, placed in liquid nitrogen cooled metal blocks, and then deposited in a Genogrinder (SPEX Sample Prep; 1 minute at 1500 rpm). A volume of 500 µL of 2x CTAB buffer (2% (v/v) CTAB, 100 mM Tris-HCl (pH 8), 20 mM ethylenediaminetetraacetic acid, 1.4 M NaCl) was added to each tube. Samples were vortexed and then incubated at 65°C for 30 minutes, inverting every 10 minutes. The samples were placed in a refrigerated tabletop centrifuge at 21,000 x *g* at 10°C for 5 minutes, and the supernatant was collected. The samples were chloroform extracted twice with an equal volume of chloroform (500 µL) and centrifuged using the same settings, keeping the supernatant after each extraction. DNA was precipitated with 0.5x volumes 5 M NaCl and 2x volumes 100% ethanol (Decon Laboratories). Samples were centrifuged using the same settings listed above. The supernatant was decanted and the DNA pellets were washed with 1 mL 70% (v/v) ethanol containing 10 mM ammonium acetate. Pellets were resuspended in 250 µL dH_2O with 10 µg/mL of Rnase A (Invitrogen) and then incubated at 55°C for 30 minutes; tubes were gently mixed halfway through. DNA was precipitated again as specified above and the supernatant was removed. Pellets were washed with 1 mL of 70% (v/v) ethanol, dried for 30 minutes at room temperature,

and resuspended in 50 µL dH_2O. DNA concentrations were checked with a Qubit (Thermo Fisher Scientific) and samples were stored at -80°C.

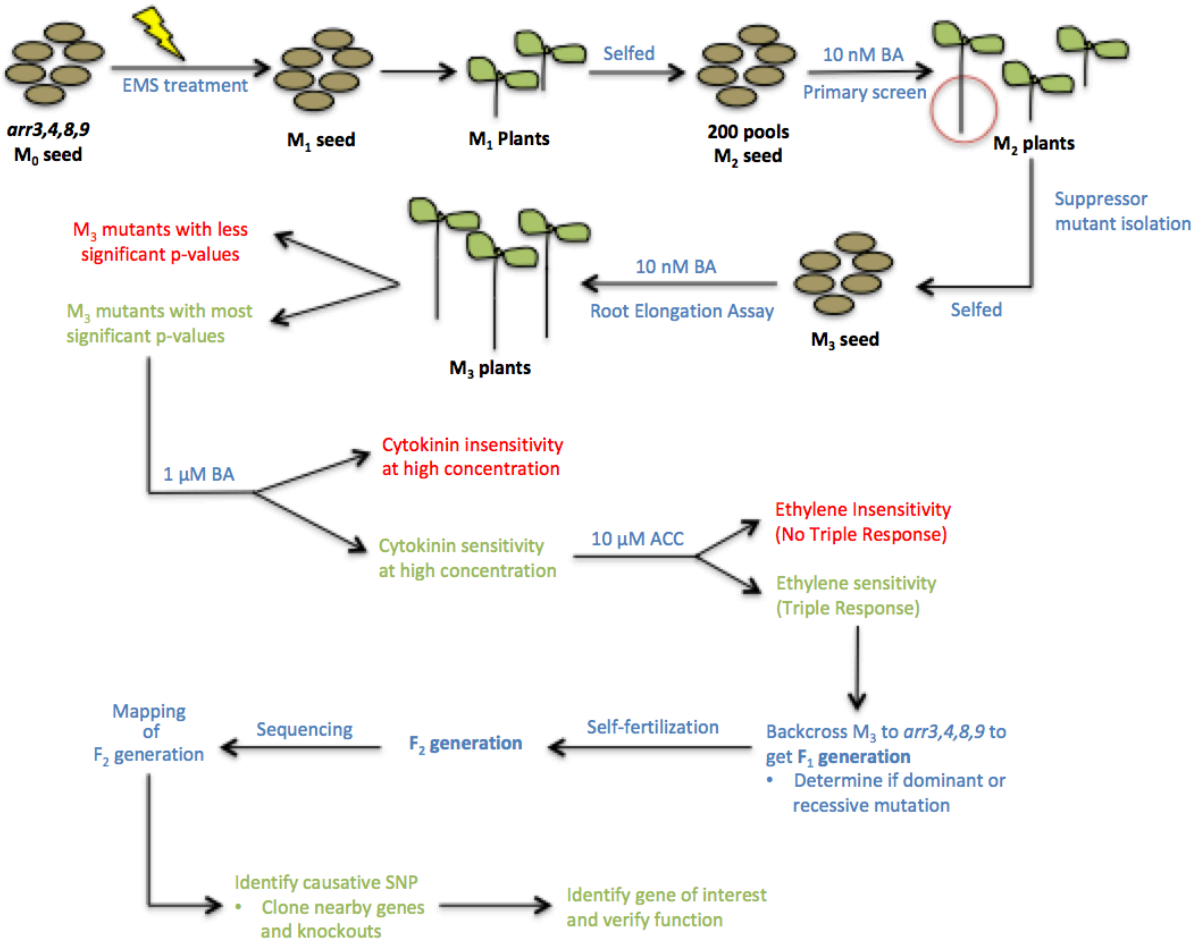

Figure 2. Project Workflow
The diagram illustrates the project workflow for isolation of putative *sch* mutants and the mapping of causal mutations.

Results

To identify candidate genes involved in the cytokinin signaling pathway, we employed a suppressor screen (Figure 2). The quadruple type-A ARR mutant (*arr3,4,8,9*), which is hypersensitive to cytokinin and displays a short root phenotype (Figure 3B), was mutagenized. M_2 lines with qualitatively longer roots than *arr3,4,8,9* were then isolated as putative

'suppressors of cytokinin hypersensitivity' (*sch*) (Figure 4). At least one putative *sch* mutant was identified for each of the 200 mutagenized M_2 pools, bringing the total number of putative *sch* mutants to 376 (Table 1).

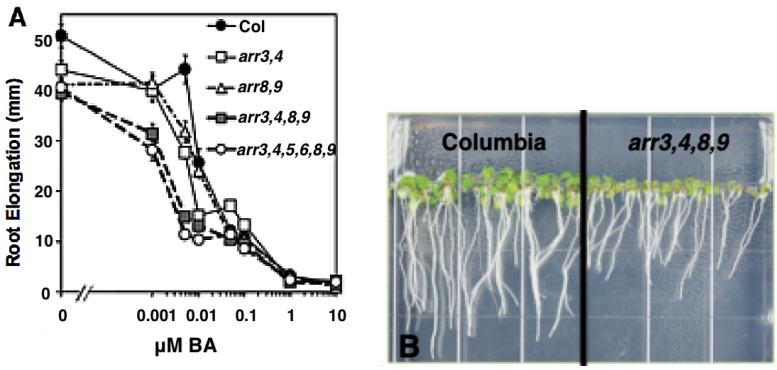

Figure 3. Hypersensitivity of *arr3,4,8,9* to low levels of cytokinin.
A) Experimental data showing root elongation of Col-0 and four different Type-A mutants in a range of BA concentrations. Mutant *arr3,4,8,9* was selected for this screen because it was highly sensitive to cytokinin at low concentrations (Figure adapted from To *et al.* 2004).
B) Col-0 and the *arr3,4,8,9* parent line were plated on 10 nM BA to demonstrate the hypersensitivity of the *arr3,4,8,9* line to cytokinin. Col-0 is shown on the left and *arr3,4,8,9* is on the right.

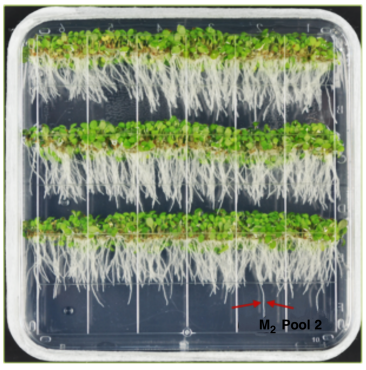

Figure 4. Isolation of putative *sch* M_2 mutants.
The pools of *arr3,4,8,9* mutants were plated on 10 nM BA and those that exhibited the long root phenotype were scored and transferred to soil. An example of a putative M_2 exhibiting a longer root is denoted by the red arrows.

The putative *sch* mutants displayed varying degrees of suppressed cytokinin hypersensitivity and enhanced root growth. Scoring of each plant was based on root length in comparison to mutants from the same pool. Plants that exhibited the greatest difference in root

length from other mutants in the pool received the highest scores. Of the putative *sch* mutants isolated, 24% scored 1 star, 32% scored 2 stars, 28% scored three stars, 12% scored four stars, and 4% scored five stars. Example putative *sch* mutants for each of the five score categories are shown in Figure 5.

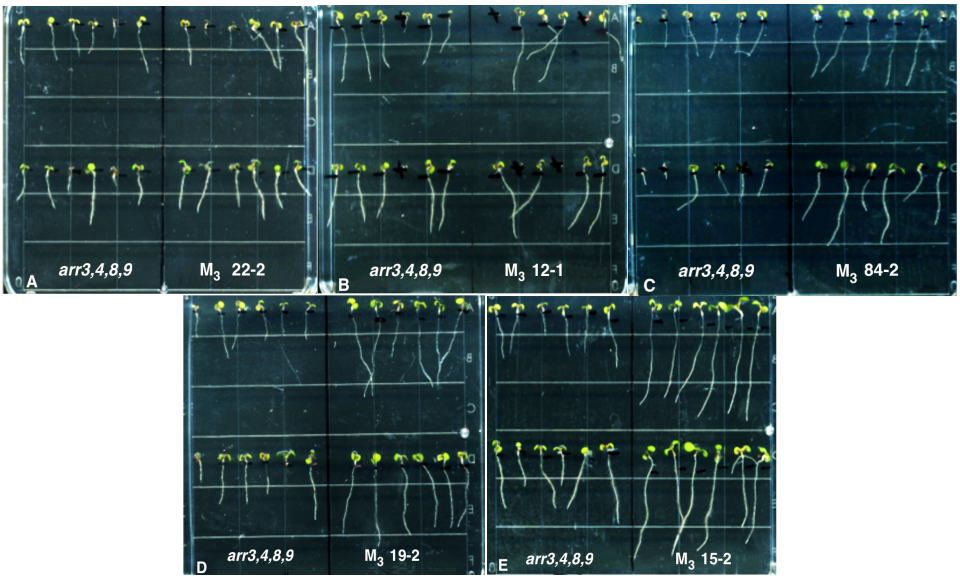

Figure 5. Validating hyposensitivity of putative *sch* M_3 lines to cytokinin. *arr3,4,8,9* seed was plated on the left and putative *sch* M_3 mutants were plated on the right. A) 1 star mutant B) 2 star mutant C) 3 star mutant D) 4 star mutant E) 5 star mutant.

With the M_2 primary screen finished, putative *sch* mutants will be verified for suppressed cytokinin sensitivity and enhanced root growth. I am now retesting the next generation of putative M_3 *sch* mutants. So far, 89 of the 180 retested putative M_3 *sch* mutants had significantly longer roots ($p < 0.05$) than those of *arr3,4,8,9* (Table 1); this was representative of 53 independent lines. Of those 89 mutants, nine of the nine tested passed a 1 μm BA test, meaning they were sensitive to cytokinin at high concentrations. Nine of the eleven tested passed a triple response assay, meaning they were ethylene sensitive and exhibited the triple response.

Table 1. Summary of screen data.

Test	Number of Mutants	Number Passed	Min Number of Independent Lines
Primary Screen	376	N/A	200
Length Score of 1	91	N/A	63
Length Score of 2	120	N/A	97
Length Score of 3	104	N/A	76
Length Score of 4	46	N/A	40
Length Score of 5	15	N/A	14
Secondary Screen	180	89	53
1 µm BA	9	9	9
Triple Response	11	9	9

Length score refers to the qualitative scoring of mutants during the primary screen, where 1 is the lowest length score and 5 is the highest.

Discussion

In this study, we report that we have identified many putative *sch* alleles that suppress cytokinin hypersensitivity. Moving forward and following the experimental setup shown in Figure 2, I will complete retests of the putative M_3 lines on low levels of cytokinin. Then, M_3 mutants with the most significant root lengths (4-5 stars and p-value < 10^{-3}) will be rescreened on an elevated level of cytokinin (1 µm BA). This should serve to screen out possible known *ahk* mutants, which are completely insensitive to cytokinin. Even at high concentrations of cytokinin, *ahk* mutants exhibit excess root elongation (Ueguchi *et al*. 2001). Thus, mutants that show complete cytokinin insensitivity will not be pursued for bulked segregant mapping, as they are likely to be *ahk* mutants.

Previous studies have also shown that ethylene-insensitive mutants can partially block the inhibitory effects of BA on root elongation (Cary *et al.* 1995). These *ethylene insensitive2* (*ein2)* mutants may be identified using a triple response assay. The triple response is characterized by a

swollen hypocotyl, an exaggerated apical hook, and shortened roots in the presence of ethylene in dark growing conditions (Guzmán and Ecker 1990). Ethylene-insensitive mutants do not exhibit the triple response when subjected to ethylene treatment. Thus, in order to test for ethylene insensitivity, putative mutants will be screened for the constitutive triple response on plates containing 10 μM ACC (a precursor of ethylene; (Cary *et al.* 1995)). Mutants that show ethylene insensitivity will not exhibit the triple response, and they will not be pursued, as our screen is focused on mutants affecting cytokinin sensitivity.

Putative M_3 *sch* mutants that pass all assays will be backcrossed to *arr3,4,8,9*. I will determine if these suppressor mutations are dominant or recessive by examining the phenotype of the F_1 seedlings on 10 nM BA plates. If the suppressor mutation is recessive, all of the F_1 seedlings will exhibit cytokinin sensitivity. If the suppressor mutation is dominant, the mutant phenotype (i.e. hyposensitivity to cytokinin) will persist into the F_1 generation. F_1 plants will be collected and allowed to self-fertilize in order to produce the F_2 generation. Mutant F_2 plants will be pooled and mapped using bulked segregant analysis. In order to acquire a final mapping interval that is smaller than the predicted density of EMS mutations, a large bulked segregant population (> 40 plants) will be utilized. This will allow us to narrow down the location of the mutation that is linked to our suppressor phenotype (Schneeburger and Weigel 2011). Once the final mapping interval is determined, I will conduct SNP analysis to identify the causative gene. Validation studies will be performed by obtaining or generating a T-DNA insertion line to verify that a loss-of-function mutation results in partial cytokinin hyposensitivity. I will also perform complementation studies to establish the number of complementation groups present among the loss-of-function *sch* mutants. If suppressor mutations are in the same gene, complementation will not occur, and the F_1 progeny will exhibit partial cytokinin hyposensitivity. If suppressor

mutations are in different genes, then the phenotype will be rescued, meaning the F_1 progeny will exhibit sensitivity to low levels of cytokinin. Thus, we will use this information to discover what genes may be functioning in the cytokinin signaling pathway. This new information will develop our understanding of the pathway and improve our ability to engineer desirable crop outcomes like increased drought tolerance and enhanced crop yields.

Acknowledgements

This project was generously funded by the National Science Foundation. I thank Dr. Kevin Potter and Duncan McPherson for mentoring me throughout my time in the lab. I also thank Dr. Joseph Kieber, Jamie Winshell, Charlie Hodgens, Dr. Asia Polko, Dr. Christian Burr, and Dr. Kwame Acheampong for their feedback on my manuscript and presentation.

References

1. Cary AJ, Liu W, and Howell SH. (1995). Cytokinin Action is Coupled to Ethylene in Its Effects on The Inhibition of Root and Hypocotyl Elongation in *Arabidopsis thaliana* Seedlings. *Plant Physiology*, 107(4): 1075-1082.
2. Deslauriers SD, Alvarez AA, Lacey RF, Binder BM, and Larsen PB. (2015). Dominant Gain-of-Function Mutations in Transmembrane Domain III of ERS1 and ETR1 Suggest a Novel Role for this Domain in Regulating the Magnitude of Ethylene Response in *Arabidopsis*. *New Phytologist*, 208: 442-455.
3. Guzmán P, Ecker JR. (1990). Exploiting the Triple Response of *Arabidopsis* to Identify Ethylene-Related Mutants. *The Plant Cell*, 2: 513-523.
4. Hutchinson C, Kieber JJ. (2002). Cytokinin Signaling. *American Society of Plant Biologists*, 14: S47–S59.

5. Kieber JJ, Schaller GE. (2014). Cytokinins. *American Society of Plant Biologists*, 12: 1-35.

6. Li X, Zhang Y. (2016). Suppressor Screens in *Arabidopsis*. *Methods in Molecular Biology,* 1363: 1–8.

7. Ma QH. (2008). Genetic Engineering of Cytokinins and Their Application to Agriculture. *Crit. Rev. Biotechnol.*, 28: 213-232.

8. Mok MC. (1994). Cytokinins and Plant Development - An Overview. In: Mok DWS, Mok MC (eds) Cytokinins. Chemistry, Activity, and Function, CRC Press, Boca Raton, FL, 155–166.

9. Reguera M, Peleg Z, Abdel-Tawab YM, Tumimbang EB, Delatorre CA, Blumwald E. (2013). Stress-Induced Cytokinin Synthesis Increases Drought Tolerance Through the Coordinated Regulation of Carbon and Nitrogen Assimilation in Rice. *Plant Physiol.*, 163: 1609-1622.

10. Schneeburger K, Weigel D. (2011). Fast-Forward Genetics Enabled by New Sequencing Technologies. *Trends Plant Sci.* 16(5): 282-288.

11. To JPC, Haberer G, Ferreira FJ, Deruére J, Mason MG, Schaller E, Alonso JM, Ecker JR, Kieber JJ. (2004). Type-A *Arabidopsis* Response Regulators Are Partially Redundant Negative Regulators of Cytokinin Signaling. *Plant Cell*, 16: 658-671.

12. To JPC, Deruére J, Maxwell BB, Morris VF, Hutchison CE, Ferreira FJ, Schaller GE, Kieber JJ. (2007). Cytokinin Regulates Type-A *Arabidopsis* Response Regulator Activity and Protein Stability via Two-Component Phosphorelay. *Plant Cell*, 19: 3901–3914.

13. Ueguchi C, Sato S, Kato T, Tabata S. (2001). The AHK4 Gene Involved in the Cytokinin-Signaling Pathway as a Direct Receptor Molecule in *Arabidopsis thaliana*. *Plant Cell Physiology*, 42: 751-755.

14. Zaman M, Kurepin LV, Catto W, Pharis RP. (2015). Enhancing Crop Yield with the Use of N-Based Fertilizers Co-Applied with Plant Hormones or Growth Regulators. *J Sci Food Agric.* 95(9): 1777-1785.

15. Zhang Y, Li X. (2005). A Putative Nucleoporin 96 Is Required for Both Basal Defense and Constitutive Resistance Responses Mediated by suppressor of *npr1-1*, constitutive 1. *The Plant Cell, 17*(4): 1306-1316.

16. Zhou A, Wang H, Walker JC, Li J. (2004). BRL1, a Leucine-Rich Repeat Receptor-Like Protein Kinase, is Functionally Redundant with BRI1 in Regulating *Arabidopsis* Brassinosteroid Signaling. *The Plant Journal*, 40: 399-409.

17. Zwack PJ, Rashotte AM. (2013). Cytokinin inhibition of leaf senescence. *Plant Signaling & Behavior,* 8: e24737.